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Molecular basis of inhibition of substrate hydrolysis by a ligand bound to the peripheral site of acetylcholinesterase

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Abstract

Acetylcholinesterase (AChE) contains a narrow and deep active site gorge with two sites of ligand binding, an acylation site (or A-site) at the base of the gorge and a peripheral site (or P-site) near the gorge entrance. The P-site contributes to the catalytic efficiency of substrate hydrolysis by transiently binding substrates on their way to the acylation site, where a short-lived acyl enzyme intermediate is produced. Ligands that bind to the A-site invariably inhibit the hydrolysis of all AChE substrates, but ligands that bind to the P-site inhibit the hydrolysis of some substrates but not others. To clarify the basis of this difference, we focus here on second order rate constants for substrate hydrolysis (k_E), a parameter that reflects the binding of ligands only to the free form of the enzyme and not to enzyme-substrate intermediates. We first describe an inhibitor competition assay that distinguishes whether a ligand is inhibiting AChE by binding to the A-site or the P-site. We then show that the P-site-specific ligand thioflavin T inhibits the hydrolysis of the rapidly hydrolyzed substrate acetylthiocholine but fails to show any inhibition of the slowly hydrolyzed substrates ATMA (3-(acetamido)-*N,N,N*-trimethylanilinium) and carbachol. We derive an expression for k_E that accounts for these observations by recognizing that the rate-limiting steps for these substrates differ. The rate-limiting step for the slow substrates is the general base-catalyzed acylation reaction k_2 , a step that is unaffected by bound thioflavin T. In contrast, the rate-limiting step for acetylthiocholine is either substrate association or substrate migration to the A-site, and these steps are blocked by bound thioflavin T.

Keywords

Acetylcholinesterase; peripheral site; thioflavin T; substrate analogs; carbamoylation; enzyme mechanism

1. Introduction

Acetylcholinesterase (AChE) 2 hydrolyzes the neurotransmitter acetylcholine at one of the highest known enzymatic rates (1). Partial inhibition of AChE activity in the brain has been

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¹The manuscript is dedicated to Professor Pelayo Camps, from the Medicinal Chemistry Laboratory of the Faculty of Pharmacy of the University of Barcelona, on the occasion of his 65th anniversary.

shown to have therapeutic benefits. AChE inhibitors that penetrate the blood-brain barrier elevate the levels of endogenous acetylcholine and are useful in the symptomatic treatment of Alzheimer's disease (2). However, complete inactivation of AChE, which can occur with organophosphate chemical warfare agents (3), can be lethal.

A better understanding of the AChE catalytic pathway may identify new mechanisms of AChE inhibition that could be therapeutically beneficial. Kinetic and thermodynamic studies have revealed that inhibitors can interact with either or both of two binding sites in AChE (4–7), and X-ray crystallography has provided information about the location of these sites (8–11). A narrow active site gorge some 20 Å deep penetrates nearly to the center of the ~65 kDa catalytic subunits. Near the base of the gorge is the acylation or A-site where H447, E334, and S203 participate in a triad that catalyzes the transient acylation and deacylation of S203 during each substrate turnover. The peripheral or P-site, spanned by residues W286 near the mouth of the gorge and D74 near a constriction at the boundary between the P-site and the A-site, specifically binds certain ligands like the neurotoxin fasciculin (12,13) and the fluorescent probes propidium (5) and thioflavin T (6). The P-site thus far has been shown to contribute to catalytic efficiency by insuring that most substrate molecules that collide with and transiently bind to the P-site proceed on to the A-site (14–16) and, with certain bound cationic substrates, by providing a modest allosteric activation of the acylation step (17).

A series of studies of substrate hydrolysis by AChE has suggested that Scheme 1 is a minimal model for the catalytic pathway (15,16). This scheme involves three enzyme substrate intermediates at the A-site (*ES*, *EAP*, *EA*) in addition to free enzyme *E*. An additional ligand corresponding to either a substrate or inhibitor can bind to the P-site (*L_P*) in any of these species, causing the rate constants for successive reaction steps involving the ternary complexes to be altered by the relative factors *a*, *g*, or *b* as indicated in Scheme 1. Formulation and analysis of the rate equation for substrate hydrolysis rates *v* that arise from Scheme 1 is a challenge, because some of the reversible reactions in Scheme 1 are not at equilibrium. In this case a general solution for *v* can be obtained only by numerical integration (16,17). Furthermore, the number of unknown parameters in Scheme 1 often exceeds the number that can be uniquely fitted to experimental data by numerical integration, allowing a number of very different sets of parameter values to fit the data equally well (17,18). To overcome this uncertainty, we have recently emphasized three overlapping strategies to simplify the analysis of AChE kinetic data (19) and yet measure parameter values unambiguously. These strategies are 1) to choose substrates that eliminate some intermediates in Scheme 1 (17), 2) to group parts of Scheme 1 that are in equilibrium (19), and 3) to determine some of the thermodynamic parameters in Scheme 1 with experiments that are independent of measurements of *v* (7).

In this report we add a fourth strategy to this list, namely to analyze the effects of AChE inhibitors on second order substrate hydrolysis rates. Classic enzymology distinguishes “competitive inhibitors” from “noncompetitive inhibitors”. Competitive inhibitors compete with substrate for a common binding site and do not alter $V_{\max}$, the maximum hydrolysis rate at saturating substrate concentration, while noncompetitive inhibitors bind to a site different from the substrate binding site and do not affect the apparent Michaelis constant K_M for substrate hydrolysis. These distinctions are of little value for AChE. Many inhibitors of AChE show “mixed” inhibition patterns in which both $V_{\max}$ and $1/K_M$ are decreased, and X-ray crystallography has revealed that these inhibitors can bind either to the A-site or the

²Abbreviations: AChE, acetylcholinesterase; ATMA, 3-(acetamido)-*N,N,N*-trimethylanilinium; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); M7C, N-methyl(7-dimethylcarbamoxy)quinolinium iodide; TcAChE, AChE from *Torpedo californica*.

³Throughout this paper we number amino acid residues according to the human AChE sequence unless otherwise noted.

P-site. A more useful classical distinction, which applies to AChE and all other enzymes, is that inhibitors which bind to the free enzyme reduce the second order hydrolysis rate constant $k_{\text{cat}}/K_{\text{M}}$, while those that bind to an enzyme-substrate intermediate reduce k_{cat} (and thereby V_{max}). To emphasize their association with the free enzyme, second order rate constants in the presence or absence of inhibitors here are denoted k_{E} (20). Scheme 1 and the corresponding rate equations for v are simplified when analysis is limited to k_{E} , and here we focus on information about inhibitor site specificity and catalytic mechanism that can be obtained from examination of k_{E} . Structure-activity relationships for ligands that bind specifically to the P-site have yet to be developed, and we reemphasize an inhibitor competition assay that can distinguish whether an inhibitor is binding to the A-site or the P-site. The impact of this assay is increased by the use of inhibitor standards whose sites of binding have been confirmed by x-ray crystallography. We then determine the effects of thioflavin T (Fig. 1), an inhibitor standard specific for the P-site, on substrate hydrolysis by acetylthiocholine and two slowly hydrolyzed analogs, ATMA and carbachol (Fig. 1), that are close structural analogs of acetylcholine. We interpret these effects in the context of substrate equilibration with AChE.

2. Experimental methods

2.1. Inhibition of substrate hydrolysis at low substrate concentrations

Recombinant human AChE was expressed as a secreted, disulfide-linked dimer in *Drosophila* S2 cells and purified as outlined previously (21). Thioflavin T (Sigma) was recrystallized from water, and concentrations were assigned by absorbance at 412 nm with $\epsilon_{412 \text{ nm}} = 36,000 \text{ M}^{-1}\text{cm}^{-1}$. ATMA was synthesized as described, and hydrolysis rates v for ATMA were determined by spectrophotometry at 290 nm ($\Delta\epsilon_{290 \text{ nm}} = 1850 \text{ M}^{-1}\text{cm}^{-1}$) (17). For acetylthiocholine, v was measured in a coupled Ellman reaction in which thiocholine generated in the presence of 0.33 mM DTNB (unless otherwise noted) was determined by formation of the thiolate dianion of DTNB at 412 nm ($\Delta\epsilon_{412 \text{ nm}} = 14,150 \text{ M}^{-1}\text{cm}^{-1}$) (17). Total AChE concentrations (E_{tot}) were calculated assuming 450 units/nmol (6). Assays were conducted at 25° C in 20 mM sodium phosphate buffer (pH 7.0), 0.02% Triton X-100 unless otherwise noted. Data fitting by nonlinear regression was conducted with SigmaPlot (vers. 11.0), and analyses with either eq. 3 or eq. 4 were weighted assuming that the dependent variable had constant percent error.

Steady-state hydrolysis of many substrates by AChE shows either a hyperbolic Michaelis-Menten or a bell-shaped Haldane dependence on substrate concentration (15). In either case, a Michaelis constant K_{M} can be calculated as a measure of the saturation of the enzyme by substrate. Since this saturation includes not just *ES* but also the other enzyme-substrate intermediates in Scheme 1, K_{M} is often generalized to K_{app} . To simplify the analysis of inhibition of substrate hydrolysis arising from the interaction of ligands with either the A- or P-sites, it is useful to focus on the second-order hydrolysis rate constant obtained when the substrate concentration $[S] \ll K_{\text{app}}$. Here we denote this constant k_{E} . In the absence of inhibitors, $k_{\text{E}} = k_{\text{cat}}/K_{\text{app}}$, where k_{cat} is the turnover number for the substrate with AChE (16). Under these conditions, the *ESS_P* complex and all downstream *ES* intermediates in Scheme 1 become negligible because $[S]$ approaches zero, and Scheme 1 reduces to Scheme 2: Scheme 2 can be applied both to inhibitors that bind at the A-site ($L_{\text{P}} = \text{I}$, and $1/i = 0$) and to inhibitors that bind at the P-site ($L_{\text{P}} = \text{I}_{\text{P}}$). Substrate hydrolysis rates v under these second-order conditions are given by eq. 1, where $[E]_{\text{tot}}$ is the total enzyme concentration.

$$v = k_{\text{E}} [E]_{\text{tot}} [S] \quad (\text{eq. 1})$$

The easiest and most accurate way to measure k_E is from the integrated form of eq. 1 at low initial substrate concentration $[S]_0$ (i.e., $[S]_0 \leq \sim 0.2K_{app}$) (22), as given in eq. 2.

$$[S] = [S]_0 e^{-zt} \quad (\text{eq. 2})$$

In eq. 2, $z = k_E[E]_{tot}$ and t is the time after mixing E and S . In addition, k_E can also be obtained by plotting reciprocal plots of $1/v$ vs. $1/[S]$ over a range of fixed $[I]$ and then replotting the slopes of these reciprocal plots vs. $[I]$ (17,22,23).

When k_2 in Scheme 2 is small enough that the substrate and inhibitor reach equilibrium with their enzyme binding site(s), an algebraic expression for v can be written (23). This expression indicates that, when an inhibitor binds to either the A- or P-sites but not both, the ratio of k_E in the absence of inhibitor ($k_{E[I]=0}$) to k_E in the presence of inhibitor (k_{E+I}) is given by eq. 3 (23).

$$\frac{k_{E[I]=0}}{k_{E+I}} = \frac{\left(1 + \frac{[I]}{K_1}\right)}{\left(1 + \frac{\alpha[I]}{K_1}\right)} \quad (\text{eq. 3})$$

Under these equilibrium conditions, α in eq. 3 is given by *ali*, the ratio of the relative acylation rate constant α and the relative affinity i of the ligands in the ternary complex in Scheme 2.

2.2 Inhibition of substrate hydrolysis in the presence of two inhibitors

Consider two inhibitors (I1 and I2), each of which show complete inhibition of second order substrate hydrolysis (i.e., $\alpha \cong 0$ in eq. 3) when analyzed individually with AChE. If a series of assays are then conducted with a fixed concentration of I1 together with varying concentrations of I2, k_E in the presence of both inhibitors (here denoted k_{E+I2}) relative to k_E when only I1 is present (here denoted $k_{E[I2]=0}$) is given by eq 4 (6).

$$\frac{k_{E+I2}}{k_{E[I2]=0}} = \frac{K_2 \left(1 + \frac{[I1]}{K_1}\right)}{K_2 \left(1 + \frac{[I1]}{K_1}\right) + [I2] \left(1 + \frac{[I1]}{K_{12}}\right)} \quad (\text{eq. 4})$$

In this equation, K_1 is the equilibrium dissociation constant for I1 with the free enzyme E , K_2 is the equilibrium dissociation constant for I2 with E , and K_{12} is the equilibrium dissociation constant for I1 with the $E I2$ complex. The value of $[I1]/K_{12}$ will be significantly greater than zero only if the ternary complex $E I1 I2$ can form, and this analysis thus can be used to detect ternary complex formation.

2.3 Inhibition of carbamoylation by a P-site ligand

With a carbamoyl ester substrate of AChE (here denoted a carbamate), both the formation and hydrolysis of the acylated enzyme intermediate, or carbamoyl enzyme (EC), are slow enough to allow equilibrium assumptions. Values of the overall carbamoylation rate constant k_{12} and the overall decarbamoylation rate constant k_{21} for a carbamate S_1 with AChE were measured by continuous recording with acetylthiocholine as a chromogenic reporter substrate S_2 that generates a hydrolysis product P_2 . Data were fitted to eq. 5, which resolves k_{12} and k_{21} (24).

$$[P_2] = \left(\frac{v_0}{k_{\text{tot}}} \right) \left[k_{21}t + \left(\frac{k_{12}}{k_{\text{tot}}} \right) (1 - e^{-k_{\text{tot}}t}) \right] + [P_2]_{t=0} \quad (\text{eq. 5})$$

In eq. 5, $k_{\text{tot}} = k_{12} + k_{21}$ and v_0 is the initial acetylthiocholine hydrolysis rate at $t = 0$ prior to the formation of any EC. An expression for k_{12} as a function of $[S_1]$ and $[S_2]$ (25) can be simplified according to Scheme 2 at low concentrations of both S_1 and S_2 , as shown in eq. 6.

$$k_{12} = \frac{k_2 [S_1] \left(1 + \frac{\alpha}{K_I} \right)}{K_M \left(1 + \frac{1}{K_I} + \frac{[S_2]}{K_{\text{app}2}} \right)} \quad (\text{eq. 6})$$

In eq. 6, $K_{\text{app}2}$ refers to K_{app} for S_2 , and k_2 and $K_M = k_{-S}k_{-1}/k_Sk_1$ are parameters for S_1 . When $[S_2]/K_{\text{app}2}$ is small, $k_{12}/[S_1]$ closely approximates k_E and eq. 3 still provides an appropriate framework for analysis of the carbamoylation rate constant k_{12} in the presence of potential inhibitors.

3. Results

3.1 Inhibition of acetylthiocholine hydrolysis

The inhibition of second order substrate hydrolysis rates provides a convenient framework for examining the interaction of an inhibitor with free AChE. Crystal structure analyses have confirmed that certain inhibitors like edrophonium (Fig. 1) bind specifically to the A-site (26), whereas others like propidium are specific for the P-site (11). Edrophonium binding to the A-site should block substrate binding to this site. In this case, no ternary complex ESI_P in Scheme 2 can form (i in this Scheme becomes very large), and α in eq. 3 is zero. This equation then predicts that k_{E+I}/k_{E+I} should have a linear dependence on the edrophonium concentration $[I]$, and this dependence was confirmed in Fig. 2A. On the other hand, propidium binding to the P-site in principle does allow formation of the ternary complex ESI_P in which S occupies the A-site, and the expected effect of propidium on k_{E+I} is less clear. However, the data in Fig. 2A clearly show that α also was zero for propidium. In fact, with the rapidly hydrolyzed substrate acetylthiocholine, k_{E+I}/k_{E+I} shows an essentially linear dependence on inhibitor concentration with virtually all inhibitors, whether they bind to the A-site (27,28), the P-site (6), or bridge both sites (27,28). Values of α in these cases are not significantly greater than zero.

3.2 Identifying the binding site for an inhibitor

The fact that α is zero for inhibitors of acetylthiocholine hydrolysis allowed us to apply a simplified inhibitor competition analysis (6,28) to determine whether a new inhibitor binds to the A- and/or P-sites. The analysis is designed to detect any ternary complex formed when both the new inhibitor and an inhibitor with a known binding site are added to AChE simultaneously. The results with thioflavin T as a new inhibitor and propidium or edrophonium as known inhibitors are illustrated in Figs. 2B and 2C. The data with propidium or edrophonium alone reflect the same measurements shown in Fig. 2A, but here plotted in the reciprocal form k_{E+I}/k_{E+I} to emphasize K_I as the IC₅₀, or inhibitor concentration at which k_{E+I}/k_{E+I} was decreased by 50%. When a fixed concentration of thioflavin T was included in the assays, the IC₅₀ for propidium was shifted to the right, indicating competition between the two inhibitors. One can calculate the extent of the shift that would result from complete competition and no ternary complex formation from the K_I values of the inhibitors individually, and these calculated shifts are shown as the dotted lines

in Figs. 2B and 2C. Propidium and thioflavin T essentially showed complete competition in Fig. 2B. The affinity of propidium or thioflavin T in the ternary complex decreased by a factor of 43 relative to their affinities in their respective binary complexes with the free enzyme, a large change that was not significantly different from complete competition at the ligand concentrations employed. In contrast, the IC₅₀ for edrophonium in Fig. 2C was not shifted by the addition of thioflavin T, indicating no competition between these two inhibitors. The relative affinities of edrophonium and thioflavin T in the ternary complex with AChE were virtually identical to those in their respective binary complexes. These data indicate that thioflavin T binds to the AChE P-site in a fashion that is essentially competitive with propidium but completely independent of edrophonium bound at the A-site. This location for thioflavin T binding has been confirmed by X-ray crystallography of the complex of thioflavin T with *Tc*AChE, which shows that thioflavin T binds adjacent to the aromatic side chains of W279, Y334 and F330 (*Tc*AChE residue numbering) in the AChE P-site (10).

3.2 Inhibition of AChE by a P-site ligand depends on the substrate

Measurement of second order substrate hydrolysis rate constants k_E at low substrate concentrations with eq. 2 has a number of advantages. The fitted constant z in this equation is the product of k_E and $[E]_{\text{tot}}$, and its value becomes indirectly dependent on the total substrate concentration $[S]_0$ only when $[S]_0$ is too high to insure strict second order hydrolysis. At lower $[S]_0$, measurement of z tolerates variation in $[S]_0$ and therefore is more accurate than measurement of an initial rate v_0 , which is proportional to $[S]_0$ as well as $[E]_{\text{tot}}$ (eq. 1) and often is not linear over the measurement interval. Furthermore, k_E is obtained from a single reaction assay, unlike slopes of reciprocal plots of $1/v$ vs. $1/[S]$ that require several assay points, and values of k_{E+I} can be collected at many more inhibitor concentrations than typically are surveyed by reciprocal plots. These features are illustrated in Fig. 3A, where the AChE-catalyzed hydrolysis of 5 μM acetylthiocholine is shown at four thioflavin T concentrations. Since K_{app} for acetylthiocholine at the ionic strength employed is 45 μM (23), the necessary condition that $[S]_0 \leq \sim 0.2K_{\text{app}}$ is met and the reaction traces are fit precisely as exponential curves as predicted by eq. 2. Analysis of k_{E+I} at various inhibitor concentrations according to eq. 3 (Fig. 3B) confirms that both thioflavin T and edrophonium inhibited acetylthiocholine hydrolysis with K_I values in good agreement with those reported in Fig. 2. Furthermore, values of α for both inhibitors were zero, again as expected from Fig. 2.

The analyses in Fig. 3 were repeated in Fig. 4 with the substrate ATMA, a cationic acetanilide that is hydrolyzed much more slowly than acetylthiocholine (k_E [$I=0$] is 700-fold lower for ATMA than for acetylthiocholine, (17)). The absorbance difference for ATMA is smaller than that for acetylthiocholine in the Ellman assay, and a somewhat higher $[S]_0$ of 10 μM (relative to the ATMA K_{app} of 47 μM (17)) was necessary for precise fitting. Nevertheless, the hydrolysis curves (Fig. 4A) again showed a good exponential correspondence to eq. 2, and values of k_{E+I} at various inhibitor concentrations could be analyzed with eq. 3 (Fig. 4B). Edrophonium inhibited ATMA hydrolysis with a K_I value in good agreement with those reported in Figs. 2 and 3, and α for edrophonium was again zero. These values strongly support the conclusions that ATMA and edrophonium compete for binding to the A-site and that ATMA binding to the A-site is on the catalytic pathway for ATMA hydrolysis by AChE. However, in contrast to its inhibition of acetylthiocholine hydrolysis, thioflavin T failed to show any inhibition of ATMA hydrolysis and gave an α value of 1.0 (Fig. 4B). We previously reported an α of 0.47 for thioflavin T inhibition of ATMA hydrolysis (17), but this value was obtained from an analysis of reciprocal plot slopes with only two concentrations of thioflavin T. The greater number of thioflavin T concentrations examined in Fig. 4B make the 1.0 value for α more reliable.

To investigate thioflavin T inhibition with another slow substrate of AChE, we turned to carbachol (Fig. 1), an isosteric carbamate analog of acetylthiocholine. The k_E for carbachol is 600,000-fold lower than that for acetylthiocholine, and the carbamoylation rate constant k_{12} can be separated from the decarbamoylation rate constant k_{21} by analysis of the approach to steady state carbamate turnover (25) (see eq. 5). This approach can be monitored by either thioflavin T fluorescence or simultaneous acetylthiocholine hydrolysis (24), and reactions monitored by acetylthiocholine hydrolysis are illustrated in Fig. 5A. Initial acetylthiocholine hydrolysis rates progressively decreased as AChE was carbamoylated until a final, relatively slow steady-state hydrolysis rate was attained. To achieve k_{12} rate constants that were second order and corresponded to eq. 6, carbachol was maintained at 0.5 mM (where $K_{app} \equiv K_M$ was 5.7 mM (24)). Acetylthiocholine concentrations were 50 μ M to minimize $[S_2]/K_{app2}$ in eq. 6. As with ATMA, thioflavin T failed to show any inhibition of carbamoylation by carbachol and gave an α value of 1.0 (Fig. 5B).

4. Discussion

4.1 With AChE substrates that reach equilibrium, bound P-site ligands do not lower the relative acylation rate constant α

The general expression for k_E in the rate equation for Scheme 2 depends on whether substrate and inhibitor binding reach equilibrium with the enzyme. Classical kinetic analyses assume such equilibria, and with this assumption α in eq. 3 is given by *ali*, the ratio of the relative acylation rate constant a and the relative affinity i for the ternary complex involving substrate and inhibitor in Scheme 2. The substrates ATMA and carbachol do equilibrate with AChE, and Figs. 4 and 5 indicate that α is 1.0 when thioflavin T is bound at the P-site in ternary complexes with both substrates. Thermodynamic data indicate that i is close to 1.0 (0.9) for the ternary complex with carbachol at the A-site and thioflavin T at the P-site (7), so a also must also be close to 1.0 for the carbamoylation step k_2 that arises from this complex. Less rigorous thermodynamic data for ATMA and thioflavin T suggested that i may be slightly greater than 1 (as much as 3) and a about 1.3 (17), but the more accurate value of α obtained here indicate that both are likely to be closer to 1.0. In no case has a ligand that binds specifically to the P-site been shown to inhibit the acylation reaction k_2 by exhibiting an $\alpha < 1$. However, an enhancement or acceleration of the acylation reaction ($\alpha > 1$) has been observed in a few cases, but only when the ligand bound at the P-site is a close analog of acetylcholine. These cases include acceleration of acylation by a second molecule of substrate at the P-site when the first substrate molecule is already at the A-site, seen with ATMA and wild-type human AChE (17), acetylthiocholine with the human W86F mutant AChE (17), and the carbamate substrate M7C with wild-type eel and human AChE (25,29). Neither substrate activation nor acceleration of acylation by another P-site ligand is seen with acetylthiocholine and wild-type [human] AChE, presumably because AChE has evolved to the extent where this acylation step is no longer rate-limiting with this highly specific substrate.

4.2 Values of the inhibition constant α provide important information about the mechanism of substrate hydrolysis

How then is the α value of zero for the ternary complex with acetylthiocholine at the A-site and thioflavin T at the P-site in Fig. 3 to be interpreted? It is extremely unlikely that α is zero for this complex for all of the reasons just cited. The answer lies in the nature of the rate-limiting step for k_E with acetylthiocholine. As emphasized by Quinn (20), the rate constant k_E monitors conversion of free enzyme and free substrate to a transition state(s) in the acylation stage of catalysis, and it incorporates all initial reversible steps in the pathway up to the first irreversible step. According to Scheme 2, the acylation reaction k_2 for AChE is virtually irreversible, but it is not necessarily the first irreversible step, i.e., the step in

which the forward reaction rate constant for an intermediate is considerably larger than the backward reaction rate constant for this intermediate. When k_2 is larger than k_{-1} and k_{-S2} in Scheme 2, bound substrate does not reach equilibrium with AChE and k_2 is no longer rate limiting. A general steady-state expression for k_E from Scheme 2 may be derived⁴, and it shows that eq. 3 involves a small approximation: The denominator of this equation includes additional $[I]$ terms. However, the limiting value of k_E $[I]=0/k_{E+I}$ at high $[I]$ is still $1/\alpha$, as predicted in eq. 3, and the corresponding expression for α is given in eq. 7.

$$\alpha = \frac{k_{S2}ak_2}{(ik_{-S2} + ak_2) \left(\frac{k_{cat}}{K_{app}} \right)} \quad (\text{eq. 7})$$

According to Scheme 2, when $[I]$ is 0, $k_E \equiv k_{cat}/K_{app} = k_S k_1 k_2 / (k_{-S} k_{-1} + k_2 k_{-S} + k_2 k_1)$ (15). When the substrate S is equilibrated with AChE ($k_2 \ll k_{-1}$ and $ak_2 \ll ik_{-S2}$), substitution into eq. 7 confirms that $\alpha = ali$, as noted above. However, when S does not reach equilibrium ($k_2 \gg k_{-1}$ and $ak_2 \gg ik_{-S2}$), then $\alpha = (k_{S2}/k_S)(k_1 + k_{-S})/k_1$. If S binding is rate limiting ($k_1 \gg k_{-S}$), $\alpha = k_{S2}/k_S$, the ratio of the association rate constant for S with EI_P to the association rate constant for S with E . This ratio is expected to be close to zero, because bound P-site ligand blocks the entry of a second ligand to the A-site by a factor of close to 100, a process that we have called steric blockade (15,23). If S migration from the P-site to the A-site is rate limiting ($k_{-S} \gg k_1$ but $k_2 \gg k_{-1}$), then $\alpha = k_{S2}(K_S/k_1)$. In this case α again would be expected to be close to zero because of the low relative value of k_{S2} .

Therefore, eq. 7 reveals how a bound P-site ligand can inhibit second order substrate hydrolysis rate constants k_E for rapidly hydrolyzed substrates like acetylthiocholine while showing no inhibition of slowly hydrolyzed substrates like ATMA or carbachol that reach equilibrium with the A-site. It remains to be determined whether substrate association or substrate migration from the P-site to the A-site is rate limiting for k_E with substrates that show some inhibition by P-site ligands like thioflavin T.

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Acetylcholine

Edrophonium

Acetylthiocholine

ATMA

Carbachol

Thioflavin T

Fig. 1.
Substrates and inhibitors of AChE.

Fig. 2.

A) Inhibition of acetylthiocholine hydrolysis by the A-site inhibitor edrophonium or the P-site inhibitor propidium. Second order rate constants k_E were calculated from data in Fig. 3 of reference (6), and the ratios $k_E [I]=0/k_E + I$ were fitted to eq. 3. For edrophonium, $K_I = 0.31 \pm 0.01 \mu\text{M}$ and $\alpha = 0.0034 \pm 0.0014$ (not significantly different from zero). Initial fitting with propidium gave $\alpha = -0.008 \pm 0.004$ (also not significantly different from zero), and α was fixed at 0 to give $K_I = 1.61 \pm 0.04 \mu\text{M}$. B, C) Inhibitor competition assay for assignment of AChE binding site specificity. Values of k_E , with inhibitors I1 (thioflavin T) and I2 as indicated, were calculated from data in Fig. 3 of reference (6), and the ratios $k_E + I_2/k_E [I_2]=0$ were fitted to the reciprocal form of eq. 4. K_2 values were set as the K_I s from Panel A, and K_1 for thioflavin T was fixed at $1.0 \mu\text{M}$ (6). For propidium and thioflavin T (panel B), the affinity in the binary complexes relative to that in the ternary complex was given by $K_{I_2}/K_1 = 43 \pm 8$. For edrophonium and thioflavin T (panel C), $K_{I_2}/K_1 = 0.81 \pm 0.04$. Dotted lines were calculated with the same values of K_1 and K_2 but with $[I_1]/K_{I_2}$ fixed at 0.

Substrate: acetylthiocholine

Fig. 3.

Inhibition of acetylthiocholine hydrolysis by the P-site inhibitor thioflavin T or the A-site inhibitor edrophonium. A) Examples of experimental traces that were analyzed with eq. 2 to obtain values of k_{E+I} . The substrate S was acetylthiocholine ($[S]_0 = 5 \mu\text{M}$), the inhibitor I was thioflavin T ($[I]$ as indicated), and $[E]_{\text{tot}}$ was 0.31 nM (for $[I] \leq 5 \mu\text{M}$) or 1.6 nM (for $[I] \geq 5 \mu\text{M}$, not shown). Traces are slightly offset because of the absorbance of thioflavin T at 412 nm. B) Values of k_{E+I} obtained from traces like those in Panel A were analyzed with eq. 3. For thioflavin T, $K_I = 1.24 \pm 0.04 \mu\text{M}$ and $\alpha = 0.0017 \pm 0.0033$ (not significantly different from zero). For edrophonium, $K_I = 0.43 \pm 0.02 \mu\text{M}$ and $\alpha = 0.0037 \pm 0.0034$ (not significantly different from zero).

Substrate: ATMA

Fig. 4.

Inhibition of ATMA hydrolysis by the A-site inhibitor edrophonium or the P-site inhibitor thioflavin T. A) Examples of experimental traces that were analyzed with eq. 2 to obtain values of k_{E+I} . The substrate S was ATMA ($[S]_0 = 10 \mu\text{M}$), the inhibitor I was edrophonium ($[I]$ as indicated), and $[E]_{\text{tot}}$ was 190 nM (for $[I] \leq 3 \mu\text{M}$) or 1900 nM (for $[I] \geq 10 \mu\text{M}$, not shown). Times zero in the traces are offset to avoid overlap. B) Values of k_{E+I} obtained from traces like those in Panel A were analyzed with eq. 3. Data for thioflavin T were averaged from two data sets, and errors were within the symbols. For edrophonium, $K_I = 0.54 \pm 0.03 \mu\text{M}$ and $\alpha = 0.0003 \pm 0.0016$ (not significantly different from zero). No K_I could be fitted for thioflavin T, so K_I was fixed at 1.24 μM (Fig. 3) to give $\alpha = 0.98 \pm 0.04$.

Fig. 5.

Thioflavin T effects on carbamoylation of AChE by carbachol monitored by acetylthiocholine hydrolysis. A) Reactions were initiated by stopped-flow mixing of AChE and thioflavin T in one syringe and carbachol, thioflavin T, acetylthiocholine, and DTNB in the second syringe (7). Final concentrations were 0.5 mM carbachol, 50 μM acetylthiocholine, 10 mM DTNB, thioflavin T as indicated, 0.5 nM AChE, 30 mM sodium phosphate, 60 mM NaCl, and 0.02% Triton X-100. Experimental traces were analyzed with eq. 5 to obtain values of k_{12} . B) Values of k_{12} from traces like those in Panel A were obtained at levels of $[S_1]$ and $[S_2]$ that were low enough to correspond to eq. 6. $[S_1]/K_{app2}$

was assumed negligible (K_{app2} in this buffer was $90\text{ }\mu\text{M}$), allowing k_{12} analysis with eq. 3. No K_I could be fitted, so K_I was fixed at $1.91\text{ }\mu\text{M}$ (24) to give $\alpha = 0.99 \pm 0.02$.

Scheme 1.

Scheme 2.